

DEMONSTRATION OF STRUCTURAL POLYMORPHISM AMONG HLA-DR LIGHT CHAINS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS*

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The HLA genetic loci in the major histocompatibility complex (MHC)¹ of man control the expression of several cell surface molecules that play a central role in intercellular communication in the immune system. The HLA-A, -B, and -C antigens are composed of a 44,000-dalton glycoprotein, which is MHC encoded, and a 12,000-dalton protein, identified as β_2 microglobulin, which does not map in the MHC (1). The HLA-D locus of the human MHC maps to the left of HLA-B on chromosome 6 (map order: centromere, D, B, C, A). HLA-D is the primary locus responsible for stimulation in the mixed lymphocyte reaction (MLR), which is thus used to define HLA-D specificities (2). A second weak MLR stimulatory locus may be linked to HLA-A (3, 4). The HLA-D locus is also linked to disease susceptibility (5) and controls immune responses to certain antigens (6). The serologically defined HLA-DR (D related) locus is closely linked or identical to the HLA-D locus. Serological typing of HLA-DRw specificities shows a strong correlation with HLA-Dw types, although the DRw typing is generally broader than that of Dw (5). In addition, HLA-DR antisera can block stimulation in the MLR (1, 7). HLA-DR controls the expression of a cell surface molecule (DR) with a more limited tissue distribution than the HLA-A, -B, and -C antigens. These DR antigens are found predominantly on B cells and monocytes, although they have also been detected on limited types of other normal and neoplastic cells (7, 8), most notably on activated T cells (9-11).

The DR molecule is composed of two noncovalently linked glycoproteins of apparent 33,000-35,000 mol wt (DR heavy or α -chain or p34) and 27,000-29,000 mol wt (DR light or β -chain or p29) (1). The HLA-D/DR region appears to be equivalent to the I region of the murine and guinea pig MHC. These I regions, which are composed of several genetic subregions, control the primary stimulatory determinants in the MLR, determine immune responsiveness to certain antigens, and control the expression of several cell surface glycoproteins (Ia antigens) similar in structure to the DR antigens (12).

Serologically, the HLA-A, -B, and -C antigens and the HLA-DR antigens are polymorphic. Within the human population, >20 alleles of the HLA-A locus and

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¹ *Abbreviations used in this paper:* 2-D, two-dimensional; fbs, fetal bovine serum; IEF, isoelectric focusing; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NET buffer, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, and 0.02% sodium azide; NP-40, Nonidet P-40; NRS, normal rabbit serum; PBS, phosphate-buffered saline; pI, isoelectric point(s); SaCl, *Staphylococcus aureus* Cowan I strain; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

>30 alleles of the HLA-B locus have been defined (5). HLA-A and -B heavy chains (44,000-dalton subunit) carrying different serologic determinants are structurally very similar to each other, displaying 80% homology in their amino acid sequences (13). It has been demonstrated that serologically different HLA-A and -B heavy chains can be distinguished by isoelectric focusing (14). By this method, each of the different heavy chains displayed microheterogeneity, in part due to a difference in sialic acid content (14). The smaller subunit, β_2 microglobulin, is invariant.

Eight alleles of the HLA-DR locus have been well established and the existence of others is indicated (5). In this study, the structural basis for the serologically detected polymorphism in HLA-DR antigens and the variability of each subunit were investigated. The DR antigens were isolated from human B lymphoblastoid cell lines homozygous for HLA-DR and were compared by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). Limited structural polymorphism was demonstrated for the murine Ia subunits using these techniques (15-17). Nonglycosylated forms of the HLA-DR polypeptide chains were also compared to determine if the carbohydrate contributed to the structural polymorphism or microheterogeneity.

Materials and Methods

Cells. Human B lymphoblastoid cell lines were obtained from the Genetics Laboratory of Oxford University, Oxford, England. The cell line 23.1 was established in this laboratory. These long-term cell lines were established by transformation of normal peripheral blood lymphocytes with Epstein-Barr virus. The HLA-Dw and -DRw specificities were determined as part of the 7th International Histocompatibility Workshop, 1977, Oxford, England. The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (fbs), 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from Grand Island Biological Co., Grand Island, N. Y.).

Preparation of Labeled Cell Extracts. Before labeling, the cells were preincubated for 1-2 h at 37°C in RPMI-1640 medium lacking methionine and supplemented with 10% dialyzed fbs, penicillin, and streptomycin to deplete the intracellular methionine pool. The cells (2×10^6) were centrifuged and resuspended at a concentration of 1×10^6 /ml in the methionine-deficient medium described above. In some experiments, cultures were supplemented with 2 μ g/ml tunicamycin (a gift from Eli Lilly and Co., Indianapolis, Ind.) and incubated at 37°C for an additional 45 min. All cultures were then supplemented with 0.25 mCi [35 S]methionine (New England Nuclear, Boston, Mass.; >500 Ci/mmol) and incubated for 12-16 h at 37°C. The labeled cells were harvested by centrifugation, washed, and added to 2×10^6 unlabeled cells. The membrane proteins were extracted by detergent solubilization in 1 ml lysis buffer containing 0.5% Nonidet P-40 (NP-40; Particle Data, Inc., Elmhurst, Ill.), 0.01 M Tris-HCl, pH 7.6, 1 mM MgCl₂, and 0.1 mM phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, Mo.). The suspension was incubated on ice for 30 min followed by centrifugation at 100,000 g for 1 h. The supernatant fraction was recovered and stored at -70°C until use.

Neuraminidase Treatment. A sample (100 μ l) of NP-40 cell extract was diluted 1:1 with 0.1 M sodium acetate, pH 5.5, 1.8% NaCl, and 0.2% CaCl₂ and divided into two aliquots. Neuraminidase (*Vibrio cholerae*, 500 U/ml; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was added to one aliquot in four additions of 5 U each, over the course of the incubation, to give a final concentration of 140 U/ml. The aliquot with and without neuraminidase was incubated at 37°C for a total of 8-9 h. The reaction was stopped by freezing the sample at -70°C before immunoprecipitation.

Antisera and Immunoprecipitation. The antisera used were all prepared in rabbits by James F. Kaufman, Harvard University, Cambridge, Mass. These included anti-p29,34 serum, which is directed against the native DR complex, and anti-p29 and anti-p34 sera, which are directed against the separated DR light and heavy chains, respectively. The immunogens were prepared

by James F. Kaufman as described elsewhere (18, 19). Briefly, the DR complex p29,34, was extracted from B lymphoblastoid cell membranes by detergent solubilization and partially purified by lentil-lectin affinity chromatography. The complex was isolated by preparative SDS-PAGE of an unboiled sample. The separated chains were isolated by preparative SDS-PAGE after boiling the sample in the presence of SDS.

The protein A-bearing bacterium *Staphylococcus aureus* Cowan I strain (SaCI) was used as an immunoadsorbent and was prepared as described by Kessler (20). The SaCI was used as a 10% (vol/vol) solution and added in a 10-fold excess (vol/vol) over the amount of antiserum used to precipitate the antigens.

The radiolabeled detergent cell extracts were pretreated for 1 h on ice with normal rabbit serum (NRS) followed by a 30-min incubation with the immunoadsorbent. This cleared the solution of biosynthetically labeled immunoglobulins and proteins adsorbing nonspecifically. The extract was centrifuged to remove the immunoadsorbent. The supernate was then incubated with the specific antiserum followed by SaCI as described above. If the anti-p29 or anti-p34 sera were to be used, the cell extract was made 1% in SDS, boiled for 5 min and cooled on ice before the incubation with the antiserum. After the incubations, the immunoadsorbent was washed extensively by repeated centrifugation and resuspension in the following buffers: two times in NET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, and 0.02% sodium azide) supplemented with 1% NP-40, 1 mg/ml ovalbumin and NaCl to give a final concentration of 0.5 M; three times in the same buffer with the NP-40 concentration reduced to 0.5%; and two times in NET buffer. The immunoprecipitated proteins were eluted from the immunoadsorbent by boiling the sample for 5 min in Laemmli gel (21) sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) containing 2% SDS followed by centrifugation to remove the SaCI.

Antibody Binding to Cell Surface. To determine whether certain proteins were present on the cell surface, antiserum was incubated with intact cells before detergent lysis and the results were compared to those obtained by immunoprecipitation from detergent extracts. 1×10^6 cells were labeled for 16 h at a concentration of 5×10^5 cells/ml with 0.25 mCi ^{35}S -methionine as described previously. The labeled cells were harvested and washed two times with Dulbecco's phosphate-buffered saline (PBS; GIBCO). The cells were resuspended in 300 μl PBS and divided into two aliquots. The control aliquot of cells was lysed by the addition of 300 μl of lysis buffer containing an extract of 3×10^6 unlabeled cells. The labeled cell extract was incubated for 30 min on ice followed by a 30 min centrifugation at 12,000 g at 4°C. The supernate was then used for immunoprecipitations using NRS and anti-p29,34 serum as described in the previous section. The experimental aliquot of intact labeled cells was incubated with 10 μl of anti-p29,34 serum for 1 h on ice. The unbound antibodies were removed by washing the cells twice with PBS and the cells were then lysed by the addition of 300 μl of lysis buffer containing 3×10^6 unlabeled cells and centrifuged as described for the control aliquot. The supernate was incubated with 100 μl SaCI to precipitate the immune complexes followed by centrifugation to remove the immunoadsorbent. The supernate of this precipitation was used for a second round of immunoprecipitation with NRS and anti-p29,34 serum. The immunoadsorbent with bound antibody-antigen complexes was treated as described in the previous section.

Gel Electrophoresis. Samples were separated according to molecular weight by discontinuous SDS-PAGE as described by Laemmli (21). The gels contained a 7-15% linear acrylamide gradient. Molecular weight standards used were bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), papain-solubilized HLA-A and -B heavy chain (34,000 mol wt), carbonic anhydrase from bovine erythrocytes (29,000 mol wt), trypsin (23,300 mol wt), and β_2 microglobulin (12,000 mol wt). Protein molecular weight markers were d.n.sylated according to the procedure of Talbot and Yphantis (22). Electrophoretic grade gel reagents were purchased from Bio-Rad Laboratories (Richmond, Calif.). Tris base and glycine were obtained from Sigma Chemical Co.

IEF was performed in slab gels of dimension 25 cm \times 10.5 cm \times 1 mm. Proteins were electrophoresed across the width of the gel. An LKB 2117 Multiphor flat bed gel apparatus (LKB Instruments, Inc., Rockville, Md.) was employed which was attached to a circulating water bath to maintain the temperature at 7°C. The IEF gels contained 8 M urea (Absolute grade; Research Plus Laboratories, Inc., Denville, N. J.), 2% NP-40 (vol/vol), 7% acrylamide, 0.19% *N,N'*-methylenebis-acrylamide, and 2% (wt/vol) Ampholine carrier ampholytes (LKB

Instruments, Inc.). The proportions of the individual ampholytes are described in the figure legends. The urea was dissolved fresh and deionized with Rexyn 300 (Fisher Scientific Co., Pittsburgh, Pa.) before use. Gels were polymerized with 0.025% ammonium persulfate and 0.0005% *N,N,N',N'*-tetramethylethylenediamine. The wick solutions were 1 M NaOH for the cathode and 1 M phosphoric acid for the anode.

Two-Dimensional Gel Electrophoresis. Two-dimensional (2-D) gel electrophoresis was carried out as described by Singer et al. (23) with modifications. In the first dimension, samples were separated by SDS-PAGE as described above. The appropriate molecular weight region was cut out of the gel. Two methods were used to excise the gel pieces depending on the type of comparison to be made.

Method A. Radiolabeled samples and dansylated protein markers were loaded in alternating wells in the SDS-polyacrylamide gel. After the gel was electrophoresed, the dansylated proteins were visualized with ultraviolet light. Each lane containing a radiolabeled sample was excised. The molecular weight region from 28,000 to 36,000 and from 41,000 to 44,000 daltons was cut out of the lane.

Method B. Alternatively, radiolabeled samples were applied to adjacent wells and dansylated protein markers were loaded in the outside wells. In this case, a band corresponding to a more limited molecular weight range was cut out horizontally across all lanes containing radiolabeled samples.

With either method, the excised gel strips were equilibrated for 2 h at room temperature in a solution of 6 M deionized urea, 2% NP-40, and 0.2% pH 9-11 Ampholines. The gel piece was then laid directly on the surface of the IEF gel along the wick at the anode. Excess solution was blotted off the gel piece and a glass strip was laid on top of it to prevent movement of the gel piece. The IEF gel was electrophoresed for 7-8 h at 1,000 V.

The pH gradient was measured with a Corning flat surface combination electrode (Corning Glass Works, Science Products Div., Corning, N. Y.). In addition, protein standards with known isoelectric points (pI) and patterns were used. These included soybean trypsin inhibitor, β -lactoglobulin, and equine and whale myoglobin.

The gel was fixed for 30 min at 60°C in 0.9 M trichloroacetic acid and 0.2 M 5-sulfosalicylic acid. Protein standards were stained at 60°C in 0.04% Coomassie Brilliant Blue G250 and 2.1% perchloric acid and destained at room temperature in 5% acetic acid, 7% ethanol, and 10% ethyl acetate (24).

The IEF and SDS-polyacrylamide gels were fluorographed as described by Bonner and Laskey (25).

Results

Comparison of DR Antigens by SDS-PAGE. Human B lymphoblastoid cell lines homozygous for HLA-D and -DR were chosen for study and are described in Table I. To compare different DR products, the cell lines were metabolically labeled with [³⁵S]methionine. The cells were lysed and membrane proteins were extracted with the nonionic detergent NP-40. The DR antigens were immunoprecipitated with a xenoserum directed against the p29,34 DR complex and analyzed by SDS-PAGE as shown in Fig. 1. The antiserum recognized four major bands. These included the DR heavy chain (p34) of an apparent 34,000-35,000 mol wt and the DR light chain (p29) with an apparent 30,000-32,000 mol wt. The heavy chain appeared to migrate similarly in all cell lines tested. However, the light chain varied in its electrophoretic mobility depending on the DR type of the cell line from which it was derived.

Two additional bands of apparent 33,000 and 32,000 mol wt and designated M1 and M2, respectively, were also precipitated with anti-p29,34 serum. Their molecular weights appeared similar in all cell lines. The proteins M1 and M2 can be dissociated from the DR complex by several methods. If the NP-40 cell extract was incubated at 37°C before immunoprecipitation, the anti-p29,34 serum precipitated mainly the DR heavy and light chain (see Fig. 4A). Alternatively, washing the immunoadsorbent-

TABLE I
HLA Types of Human B Lymphoblastoid Cell Lines

Cell line	HLA type				
	A	B	Cw	Dw*	DRw‡
IBW4	3,3	35,35	4	1,1	1,1
METTE	2,3	5,18		1,1	1,(1)
MST	3,3	7,7		2,2	2,2
PGF	3,3	7,7		2,2	2,2
LKT	1,1	8,8		3,3	3,3
WT20	30,30	18,18	5	0	3,3
PRIESS	2,2	15,15	3	4,4	4,4
MICH	2,32	27,15	2	0	5,(5)
ARENT	2,2	38,39		6,6	6,(6)
MANN	29,29	12,12	4	7,7	7,7
JMF	23,23	12,12	4	7,7	7,(7)
MADURA	2,2	40,40	3	8,8	8,8
23.1§	2,2	27,27	1	8,8	8,8

* 0 indicates not typed.

‡ The numbers within parentheses indicate preliminary typing.

§ The donor for this cell line was typed by Dr. B. G. Solheim, Tissue Typing Laboratory, University Hospital, Oslo, Norway.

bound antibody-DR antigen complex with 0.1% SDS removed most of M1 (data not shown).

Actin has been shown to be a major component of purified human lymphocyte membranes and is a common contaminant of immunoprecipitates from lymphocyte extracts (26, 27). In Fig. 1, the band with apparent 44,000 mol wt migrated as expected for actin (42,000–44,000 daltons) and it had a similar pI as will be shown in the next section. It was seen in all immunoprecipitations including those using NRS.

It has been demonstrated that the heavy and light chains of DR antigens are cell surface glycoproteins. To determine if M1 and M2 are accessible at the cell surface, intact radiolabeled cells were incubated with anti-p29,34 serum as described in Methods. After this incubation, the unbound antibody was removed by washing the cells which were then lysed in the presence of a sixfold excess of unlabeled cells to prevent the immunoprecipitation of newly accessible intracellular proteins. The immune complexes were precipitated with SaCl. The results are presented in Fig. 2. Lane 2 demonstrates that when the antibody has access only to the outside of the cell, the predominant proteins recognized are the DR heavy and light chains and M2 but not M1. After the incubation of the antiserum with the intact cells, detergent lysis, and precipitation with SaCl, the supernate was used for a second round of immunoprecipitation with anti-p29,34 serum to reveal possible intracellular proteins. Lane 3 (Fig. 2) shows that M1 and some additional heavy and light chains are precipitated from the detergent cell extract after removal of the cell surface DR antigens.

Comparison of DR Antigens by 2-D Gel Electrophoresis. The extent of structural polymorphism among allelic products of HLA-DR was further investigated by 2-D gel electrophoresis. In this procedure, SDS-PAGE was used in the first dimension and IEF was used in the second dimension. Because most of the proteins of interest were not water soluble, NP-40 was included in the IEF gel. This prevented the direct application of the SDS-polyacrylamide gel strips at the cathode of the IEF gel as

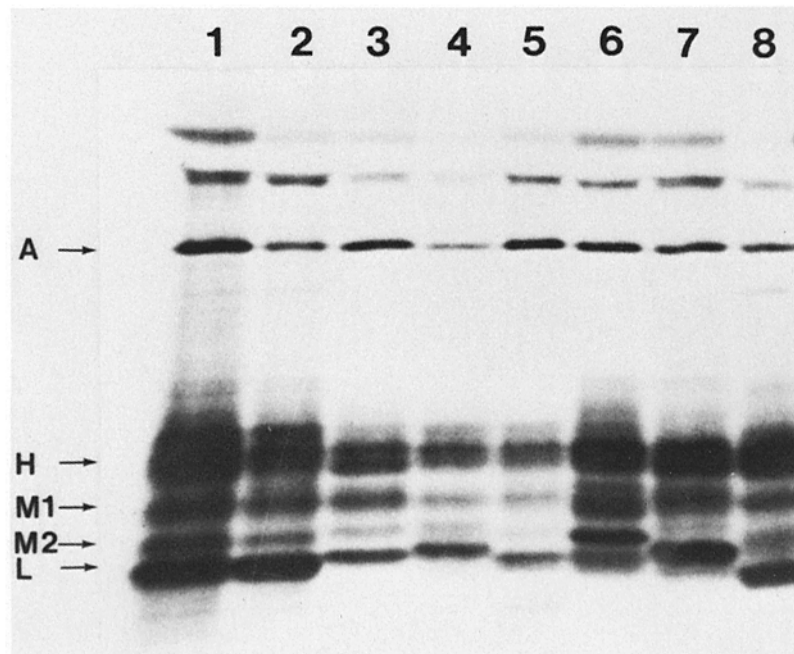


FIG. 1. Fluorograph of SDS-polyacrylamide gel of DR antigens which were immunoprecipitated from NP-40 extracts of [35 S]methionine-labeled cell lines using anti-p29,34 serum. The cell lines used were IBW4 (lane 1), MST (lane 2), LKT (lane 3), PRIESS (lane 4), MICH (lane 5), ARENT (lane 6), MANN (lane 7) and MADURA (lane 8). The lane number in this figure corresponds to the DRw type of the cell line. The indicated bands are A, actin; H, DR heavy chain; and L, DR light chain. M1 and M2 are described in the text.

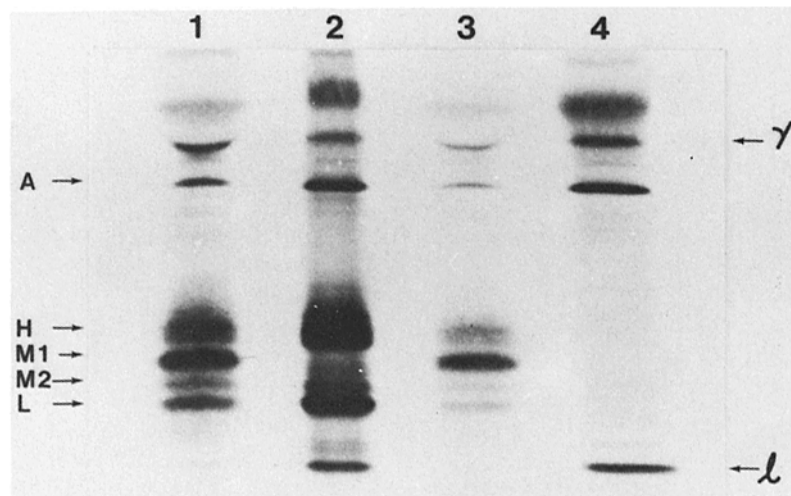


FIG. 2. Fluorograph of SDS-polyacrylamide gel demonstrating accessibility of DR antigens at cell surface. Lane 1: control immunoprecipitate from cell extract using anti-p29,34 serum. Lane 2: intact cells were incubated with anti-p29,34 serum and then lysed in the presence of unlabeled cells and the immune complexes were precipitated with SaCl (experimental immunoprecipitate). Lane 3: immunoprecipitate from cell extract using anti-p29,34 serum after removal of the cell surface antigens as described for lane 2. Lane 4: immunoprecipitation from cell extract using NRS. The cell line used was LKT. The indicated bands are IgG heavy (γ) and light (l) chains. The other bands are described in the legend to Fig. 1.

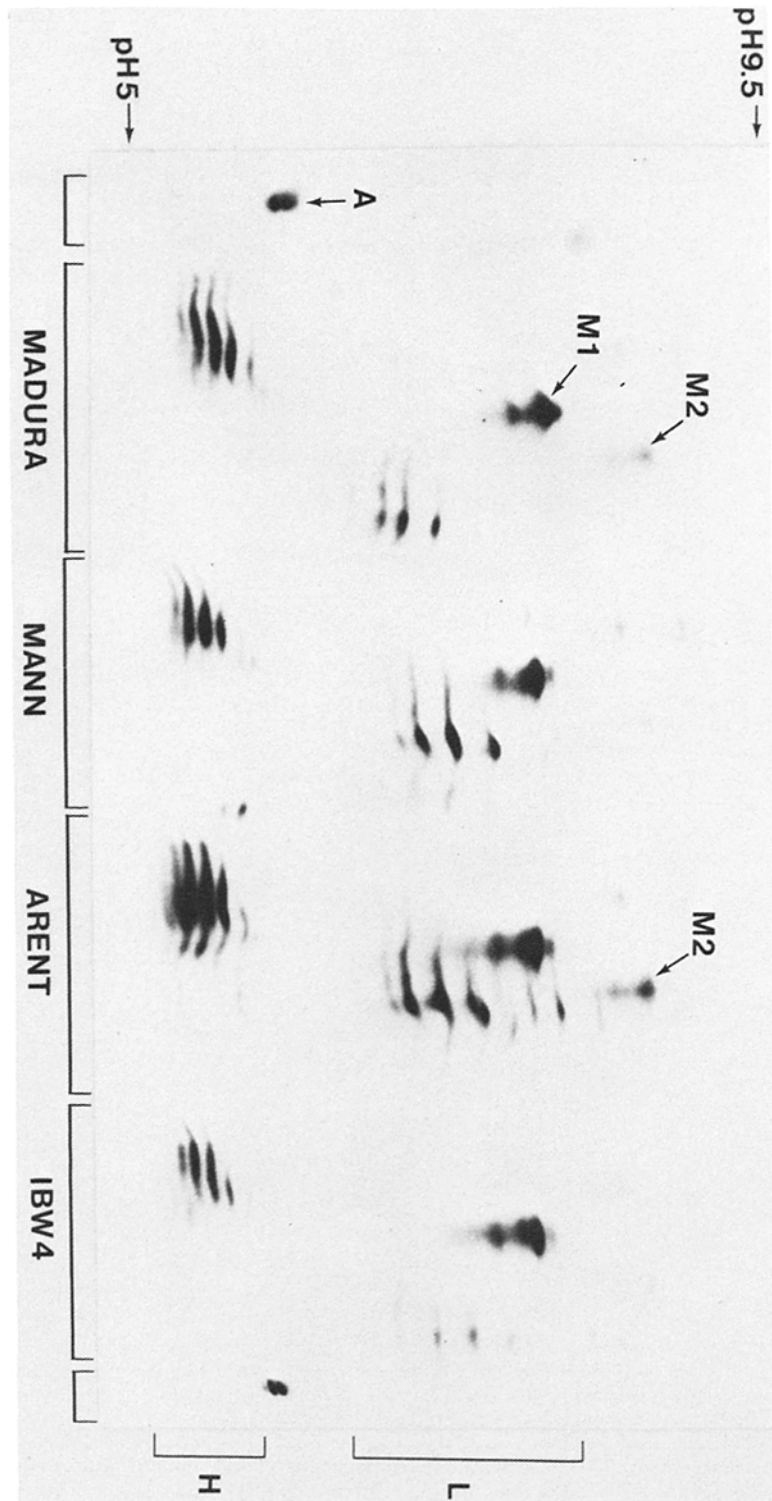


Fig. 3. Fluorographs of 2-D gels of DR antigens from different cell lines. The antigens were immunoprecipitated with anti-p29.34 serum from labeled cell extracts and electrophoresed as described in Materials and Methods and the text. The brackets beneath the gels indicate the placement of the individual gel pieces at the anode (+) of the IEF gel. Each gel piece was placed with the higher molecular weight end to the left. The brackets at the side indicate the pH range over which the DR heavy (H) and light (L) chains focus. The proteins M1 and M2 are present in all immunoprecipitates but are indicated by arrows in only a few. The total concentration of ampholytes in the IEF gel was 2.3% (wt/vol) in proportions of 78% pH 3.5-10, 6% pH 9-11, 6% pH 6-8, 6% pH 5-7, and 4% pH 3.5-5 Ampholytes.

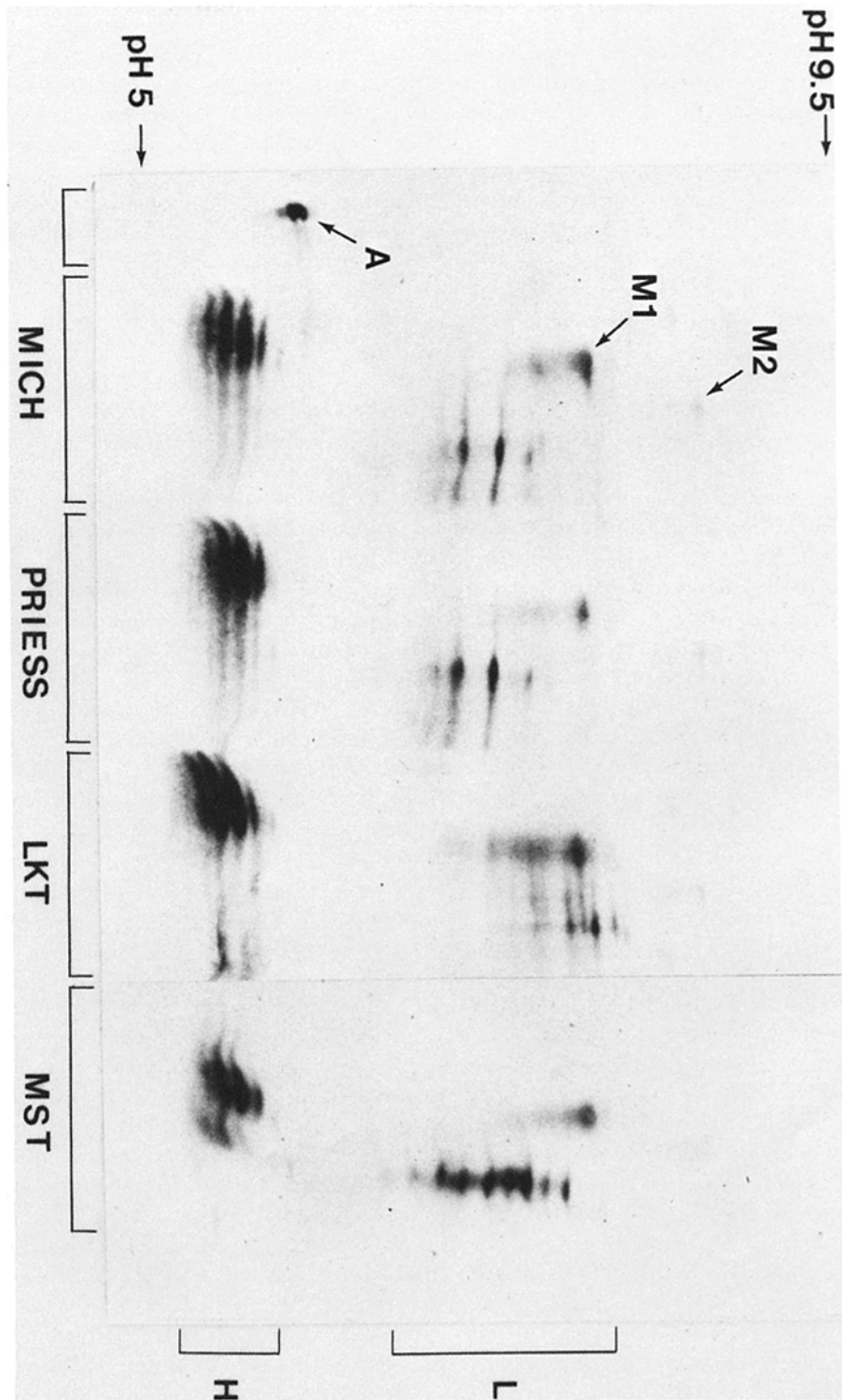


FIG. 3, continued.

described by Singer et al. (23), because the SDS apparently formed mixed micelles with the NP-40 which migrated to the anode, effectively clearing the IEF gel of detergent. It was found that equilibrating the SDS-polyacrylamide gel strips in 6 M urea and 2% NP-40 and applying the strips at the anode overcame this problem.

Fig. 3 shows a comparison of the 2-D gel patterns of the DR antigens immunoprecipitated from eight cell lines. After the first-dimension gel was electrophoresed, the lanes were cut out as described for method A in the region corresponding to 28,000–36,000 daltons. Limiting the area of the SDS-polyacrylamide gel excised allowed the application of multiple samples to one IEF gel facilitating the direct comparison of several cell lines. The area corresponding to actin (41,000–44,000 daltons) was also cut out of the gel because this protein provided a reference point in the second dimension.

Several structural features of the DR antigens are revealed by comparing different specificities by 2-D gels. Both the DR heavy and light chains focused as multiple bands, possibly caused by heterogeneity in post-translational modifications such as glycosylation or phosphorylation. The heavy chain focused as a cluster of three to four prominent bands around pI 5.4–5.7. No major differences in the pattern were noted among all cell lines tested. In contrast, the DR light chains generated IEF patterns consisting of three to seven major bands ranging in pI from 6.5 to 8. The pattern varied considerably among cell lines expressing different DRw specificities. Therefore, although both the DR heavy and light chains display microheterogeneity within the product of a single allele, only the light chain shows structural polymorphism among the products of different alleles.

The DRw 4 specificity cross-reacts serologically with DRw 5 and 7 specificities (5) and the 2-D gel patterns of these three DR light chains also show similarities. The DR light chains isolated from the cell lines PRIESS (DRw4), MICH (DRw5), and MANN (DRw7) each focused as a band at pI 7, two major bands at pI 6.8 and 6.6, and a minor, more acidic band. A second minor spot at pI 7 was apparent for the DRw 5 light chain but not for DRw 4 or 7. The 2-D pattern of the light chain from MADURA (DRw8) was similar to that of DRw 7 but shifted to a more acidic pH.

The light chains from the cell lines ARENT (DRw6) and MST (DRw2) both exhibited a complex 2-D pattern consisting of at least seven bands. The DRw 6 light chain pattern appeared to consist of at least two clusters of bands; one cluster of three to four bands resembled the DRw 7 light chain pattern and the second cluster of four spots was more basic and less intensely labeled. The most basic light chain was that isolated from LKT (DRw3) which had a cluster of about four spots at a pI of 7.3–7.6.

The 2-D gel pattern of several cell lines expressing the same DRw specificity were compared to look for differences not correlating with the HLA-D/DR type. This type of analysis revealed no major differences in the DR heavy and light chains among cell lines sharing DRw specificities whether or not they shared HLA-A, -B, and -C specificities. For example, DR antigens isolated from IBW4 and METTE (DRw1) generated the same 2-D pattern, as did MST and PGF (DRw2), LKT and WT20 (DRw3), MANN and JMF (DRw7), and MADURA and 23.1 (DRw8) (data not shown).

The protein M1 had a pI of ~7.4. It appeared as one major spot but may be resolved into more than one band (Fig. 3, MANN). The M2 protein appeared as a faint spot at pI 8.3. It was most evident in the 2-D gel pattern of ARENT (Fig. 3), but

it was present in all cell lines. The M1 and M2 proteins appeared invariant in all cell lines tested.

The band (44,000 daltons) identified as actin from the first-dimension gel focused as two closely spaced spots at \sim pI 5.8. It has been shown that there are two forms of non-muscle cell actin which can be distinguished by IEF and have a pI of 5.4 (28).

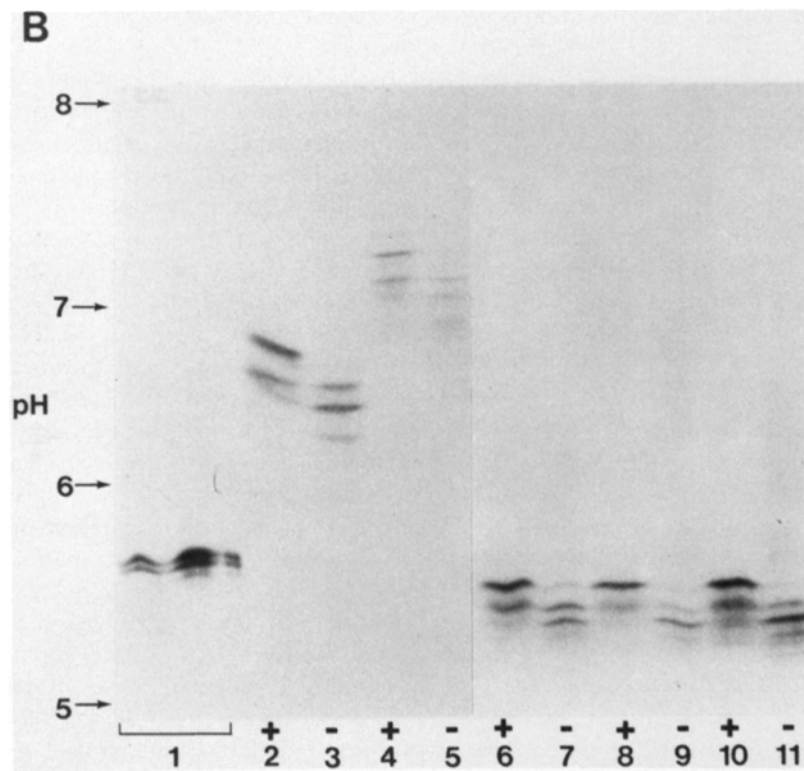
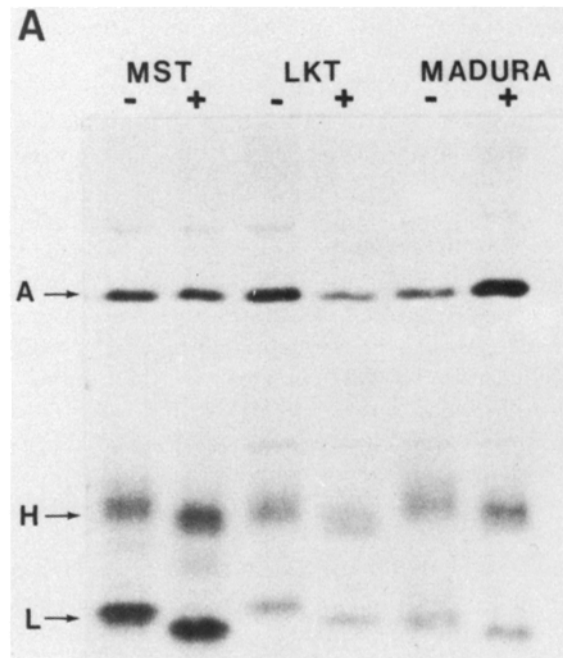
Neuraminidase Treatment of DR Antigens. The DR light and heavy chains have an average sialic acid content of 2.2 and 2.6 mol per mol respectively (29). In Fig. 3, the increasing acidity of the heavy chain bands was accompanied by an increasing apparent molecular weight consistent with an increasing sialic acid content. To further investigate the source of the microheterogeneity within each allele of the DR heavy and light chains, labeled cell extracts were incubated with neuraminidase to remove terminal sialic acids. The DR antigens were then immunoprecipitated with anti-p29,34 serum and separated by SDS-PAGE. Fig. 4A demonstrates that after incubation with neuraminidase, the apparent molecular weights of both the DR heavy and light chains decreased, consistent with the removal of sialic acids. As noted earlier, incubation at 37°C in the presence or absence of the enzyme leads to the disappearance of most of M1 and M2.

Fig. 4B shows the 2-D gel patterns of several DR antigens after incubation at 37°C with or without neuraminidase. In this experiment, bands were excised from the SDS-polyacrylamide gel as described in method B. A band corresponding to 36,000–32,000 daltons was cut out across several lanes, as was the subjacent band corresponding to 32,000–29,000 daltons, and both were applied to the IEF gel. This procedure allowed a more direct comparison of polypeptides of a given molecular weight from different cell lines.

After neuraminidase treatment the DR heavy chain was shifted to a more basic pI consistent with the removal of sialic acids. However, even after an extensive treatment with neuraminidase, the heavy chain pattern was not collapsed to a single band. A similar pattern was observed for all cell lines analyzed. The DR light chain also became more basic after neuraminidase treatment, and did not collapse into a single band. The light chains of different DRw specificities still differed in their pI, despite removal of sialic acids. Thus sialic acid differences cannot account for structural polymorphism among the products of different HLA-DR alleles.

Effect of Tunicamycin on DR Antigens. The structural polymorphism between light chains of different DR specificities could be a result of differences in amino acid sequence or in the oligosaccharide moieties. In the last section, it was demonstrated that some of the microheterogeneity within, but not polymorphism among, allelic specificities was a result of variability in the number of sialic acid residues. To study the role of the rest of the carbohydrate in determining structural polymorphism, cell lines were metabolically labeled in the presence of tunicamycin. Tunicamycin prevents the addition of *N*-linked carbohydrate to protein by inhibiting the formation of *N*-acetylglucosamine-lipid intermediates (30, 31).

Figs. 5 and 6 show that under the conditions used, the amounts of glycosylated DR heavy and light chains were reduced and new bands appeared at \sim 29,000 and 27,000–28,000 daltons. These bands are presumably nonglycosylated forms of the DR polypeptides. Xenoantisera directed against the separated heavy and light chains were used to analyze the relationship of these nonglycosylated chains to their glycosylated counterparts. Anti-p34 serum precipitated the 34,000-dalton glycosylated



heavy chain, as expected (Fig. 5, lane 3), and also recognized the new 29,000-dalton polypeptide (Fig. 5, lanes 8–15). This new species, isolated from eight different cell lines, migrated identically (Fig. 5). The anti-p29 serum precipitated the glycosylated light chain (30,000–32,000 mol wt, Fig. 5, lane 2 and Fig. 6) and the new band migrating at 27,000–28,000 daltons (Fig. 5, lanes 5, 7 and Fig. 6). Some variability in the electrophoretic mobility of this new protein was observed when different DRw specificities were compared (Fig. 6, lanes 5–8). Thus tunicamycin appears to convert the DR heavy chain to a species of apparent 29,000 mol wt and the light chain to a species of apparent 27,000–28,000 mol wt. It was noted that the anti-p29 serum could also precipitate some of the DR heavy chain (Fig. 5, lanes 2, 7). This was probably a result of incomplete separation of the chains because it was not consistently observed (Fig. 6). In some cell lines, additional bands between 27,000 and 30,000 daltons were precipitated by anti-p29,34 serum, but these were not recognized by antisera against the separated chains.

The glycosylated and nonglycosylated forms of the DR antigens were compared by 2-D gels. The antigens precipitated from one cell line by anti-p29,34 serum were cut out of the first-dimension gel by method A to include the molecular weight range of 24,000 to 36,000 daltons and then analyzed by IEF. Fig. 7 A shows that after removal of the *N*-linked glycan chains, the heavy and light chains each became more basic and focused as two bands.

The DR antigens from tunicamycin-treated cells were also precipitated with anti-p29 or anti-p34 sera after boiling in SDS to separate the chains as described earlier. The immunoprecipitated proteins were separated in the first dimension by SDS-PAGE and excised for IEF as described in method B. For analysis of the nonglycosylated heavy chain, a band corresponding to ~27,000–30,000 daltons was cut out across the lanes containing immunoprecipitates from different cell lines. The IEF pattern of the nonglycosylated heavy chain (Fig. 7 B) demonstrates that no variability in pI can be detected among the cell lines analyzed.

Fig. 7 C shows the IEF pattern of the nonglycosylated forms of the DR light chains. In this case a band from ~26,000–29,000 daltons (nonglycosylated light chain) and a band from 29,000–32,000 daltons (glycosylated light chain) were excised from the SDS-polyacrylamide gel by method B and applied to the IEF gel. It is evident that the DR light chains are still structurally polymorphic after treatment with tunicamycin. The light chains from the eight cell lines analyzed form four pI groups which are defined by (a) MADURA (DRw8), (b) PRIESS (DRw7), MICH (DRw5), and MANN (DRw7), (c) IBW4 (DRw1) and MST (DRw2), and (d) LKT (DRw3) and ARENT (DRw6). Within each group the DRw specificities are serologically cross-reactive (5). However, not all cross-reactive specificities show structural similarities. For example, DRw2 and 6 are strongly cross-reactive serologically, but their light chains have very different pI. It is apparent from Fig. 7 that the nonglycosylated DR

FIG. 4. Fluorographs of one-dimensional and 2-D gels of neuraminidase-treated DR antigens. (A) Labeled cell extracts were incubated at 37°C in the presence (+) or absence (–) of neuraminidase followed by immunoprecipitation with anti-p29,34 serum and analysis by SDS-PAGE. (B) 2-D gel of samples from (A). Lane 1 is actin. Lanes 2–5 are DR light chains and lanes 6–11 are DR heavy chains incubated with (+) or without (–) neuraminidase. The cell lines used were MADURA (lanes 2, 3, 6, 7), LKT (lanes 4, 5, 8, 9), and MST (lanes 10, 11). The concentration of ampholytes was 2% (wt/vol; 40% pH 3.5–10, 30% pH 6–8, and 30% pH 4–6).

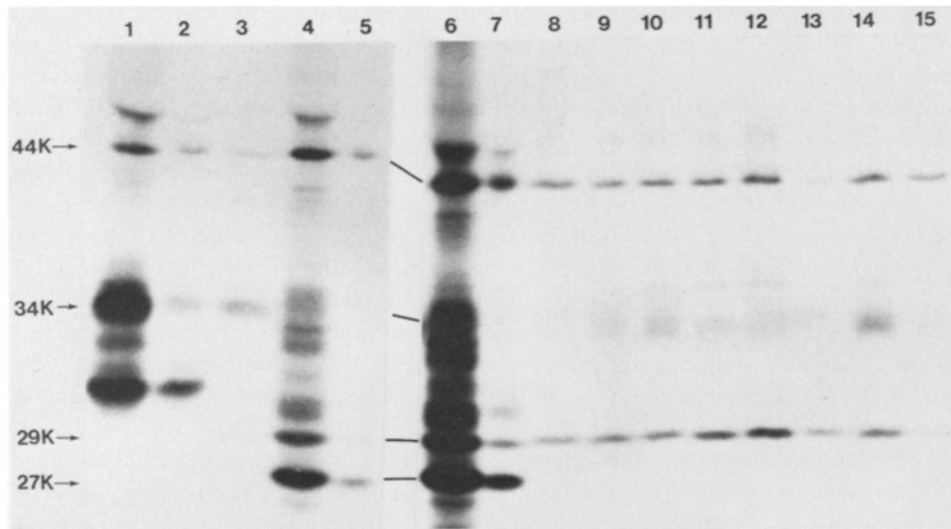


FIG. 5. Fluorograph of SDS-polyacrylamide gel of DR antigens from normal or tunicamycin-treated cells. Lanes 1-3 are immunoprecipitates from an untreated, labeled PRIESS cell extract using anti-p29,34 (lane 1), anti-p29 (lane 2), or anti-p34 (lane 3) sera. Lanes 4-15 are immunoprecipitates from NP-40 extracts of tunicamycin-treated cells using anti-p29,34 (lane 4, 6), anti-p29 (lane 5, 7), or anti-p34 (lanes 8-15) sera. The cell lines used were IBW4 (lanes 4-8), MST (lane 9), LKT (lane 10), PRIESS (lane 11), MICH (lane 12), ARENT (lane 13), MANN (lane 14), and MADURA (lane 15). Lanes 6-15 were exposed twice as long as lanes 1-5. Lanes 4 and 6 and lanes 5 and 7 are equivalent.

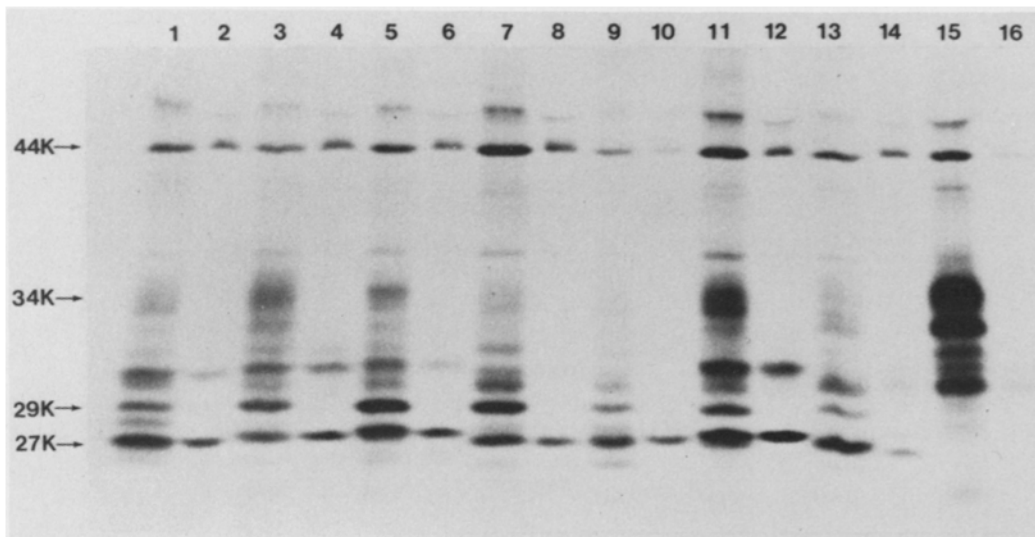


FIG. 6. Fluorograph of SDS-polyacrylamide gel of DR antigens from normal (lanes 15, 16) and tunicamycin-treated (lanes 1-14) cells. Cell extracts were immunoprecipitated with anti-p29,34 serum (lanes 1, 3, 5, 7, 9, 11, 13, 15) or anti-p29 serum (lanes 2, 4, 6, 8, 10, 12, 14, 16). The cell lines used were MST (lanes 1, 2), LKT (lanes 3, 4), PRIESS (lanes 5, 6), MICH (lanes 7, 8), ARENT (lanes 9, 10), MANN (lanes 11, 12), and MADURA (lanes 13-16).

heavy and light chains of each specificity still display microheterogeneity. The bands of each doublet are separated by 0.1–0.2 pH units. Other minor bands are also apparent upon close inspection. The source of this heterogeneity is being further investigated.

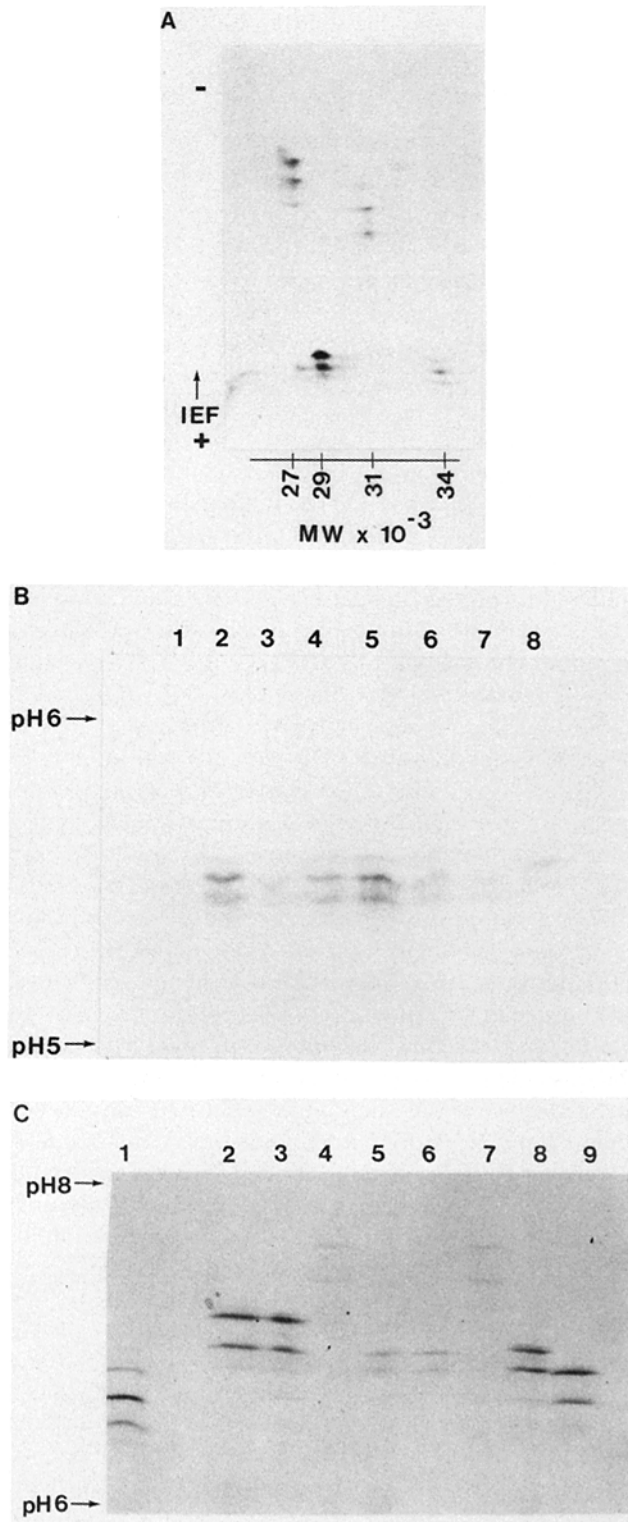
Discussion

The data presented in this paper demonstrate that the light chains of DR antigens which differ serologically are structurally polymorphic. However, no definable differences were detected among the DR heavy chains. These results agree with those of Silver and Ferrone (32) who compared the [³H]phenylalanine-labeled tryptic peptides of DR antigens from the B cell lines Victor (DRw4,6) and WIL 2 (DRw4,7). IEF of the peptides revealed major similarities in the heavy (α) chains, whereas the light (β) chains were strikingly different. These results have been confirmed and extended by comparison of the tryptic peptide maps of DR antigens from four other cell lines using ion exchange chromatography (J. F. Kaufman, R. A. Andersen, and J. L. Strominger. Manuscript in preparation.). The lysine-labeled peptides of the DR heavy chains were virtually identical whereas the peptides of different DR light chains were dissimilar.

Contrary results were reported by Klareskog et al. (33) who found more structural variation in the heavy chains than in the light chains. This was based on a comparison by high pressure liquid chromatography (HPLC) of the [³H]tyrosine-labeled tryptic peptides of DR antigens from the cell lines Daudi (DRw6, unidentified) and Raji (DRw3,6). The source of the discrepancy is not apparent.

This analysis of the variability of the DR subunits was undertaken to determine the relationship of protein structure to the serological polymorphism of HLA-DR in the human population. As demonstrated in this study, the DR light chains from cell lines with the same DR type exhibited the same 2-D pattern, whereas the light chains of different DRw specificities showed different patterns. These findings were independent of the HLA-A, -B, and -C type but correlate with the HLA-D/DR region type. Furthermore, some serologically cross-reactive DR types generally showed related 2-D gel patterns of their light chains, although this correlation was not absolute. Because limited or no structural polymorphism has been demonstrated for the DR heavy chain, the structural polymorphism observed here for the DR light chain may be the basis of the serologically detected polymorphism.

Other approaches to answer the question of which DR subunit carries the alloantigenic determinants have generally led to ambiguous conclusions. In these studies the DR chains were separated and independently reacted with alloantisera or xenoantisera. Generally, xenoantisera recognized both subunits although the degree of binding varied. Snary et al. (34) reported preferential precipitation of the light chain with xenoantiserum. However, other laboratories (33, 35, 36) were able to bind a proportion of heavy chain as well as light chain. An early report using human alloantisera found preferential binding to the small subunit (36). Recently, Tosi et al. (35) found no significant binding of the heavy chain to alloantisera, whereas the light chain was recognized by some sera with a maximum binding of 20%. However, Klareskog et al. (33) reported the opposite results. As a result of the extensive denaturing conditions used (SDS, 56°C, or urea) in most of the experiments cited above, it can not be concluded that a DR subunit that does not bind antisera does



not carry an alloantigenic determinant. A different approach was taken by Barnstable et al. (37) who studied the expression of DR antigens on human-mouse somatic cell hybrids. In their study, the hybrids did not react with xenogeneic DR antiserum but a few clones did react with alloantisera by immunofluorescence. Because the xenoserum reacted predominantly with the small subunit, they concluded that the hybrids lacked the light chain, and that the alloreactivity was a result of expression of the large subunit. However, they did not show that the alloantisera did, in fact, bind to a DR heavy chain.

Most studies investigating the alloantigenic determinants of the DR and Ia molecules have concluded that the protein and not the carbohydrate portion of the molecule determines alloreactivity. Enzymatic removal of the glycan chains from the DR subunits leaves the alloantigenic activity intact (38). Similar studies on the murine Ia molecule have shown that after removal of the carbohydrate, the two chains are still precipitable with alloantisera, whereas digestion of the molecule with pronase yields glycopeptides which do not appear to bind alloantibody (39). The present study supports the conclusions of these studies because it was demonstrated that the DR light chains are still structurally polymorphic despite the removal of sialic acid or *N*-linked oligosaccharide. Preliminary evidence also indicates that the tunicamycin-treated DR molecules are precipitable with alloantisera ([40], H. Ploegh. Personal communication.). However, tunicamycin only prevents the addition of *N*-linked oligosaccharide to protein. Nishikawa et al. (41) found that although tunicamycin treatment of a B-cell line reduced the molecular weights of the DR heavy and light chains, consistent with the removal of a glycan chain, the cells were still able to incorporate [³H]glucosamine into the large subunit. This observation suggests that the heavy chain may contain another type of oligosaccharide in addition to an *N*-linked one.

The 2-D gel pattern of the DR heavy chain observed in this study is similar to that observed by Ikeman et al. (42) who reported that the large subunit from different cell lines all focused as a cluster of four spots within a pH range of 5.3–5.4 and corresponding to a mol wt of 33,000. The antigens isolated from chronic lymphocytic leukemia cells showed additional, more basic spots at a higher molecular weight, but it was not demonstrated that these represent heavy chain variants. Springer et al. (19) reported that the DR heavy and light chains isolated from the cell line 4265 had pI of 5.2 and 6.1, respectively. In this study, the pI assigned to the various bands are tentative because it was not determined if the proteins had reached an equilibrium position. The pI are reported to facilitate comparisons.

FIG. 7. Fluorograph of 2-D gels of DR heavy and light chains from tunicamycin-treated cells. (A) 2-D gel showing the glycosylated DR heavy (34,000 mol wt) and light (31,000 mol wt) chains and the nonglycosylated heavy (29,000 mol wt) and light (27,000 mol wt) chains. The NP-40 extracts of tunicamycin-treated PRIESS cells were immunoprecipitated with anti-p29,34 serum. A single lane from the SDS gel was excised and analyzed on the IEF gel. The proportions of ampholytes used were the same as given in Fig. 3. (B) 2-D gel of nonglycosylated DR heavy chains isolated with anti-p34 serum from extracts of tunicamycin-treated cells. The cell lines used were MADURA (lane 1), MANN (lane 2), ARENT (lane 3), MICH (lane 4), PRIESS (lane 5), LKT (lane 6), MST (lane 7), and IBW4 (lane 8). The IEF gel contained 2% (wt/vol) ampholytes composed of 40% pH 3.5–10 and 60% pH 4–6 Ampholines. (C) 2-D gel of glycosylated (lane 1) and nonglycosylated (lanes 2–9) DR light chains isolated with anti-p29 serum. The cell lines are IBW4 (lane 2), MST (lane 3), LKT (lane 4), PRIESS (lanes 1, 5), MICH (lane 6), ARENT (lane 7), MANN (lane 8), and MADURA (lane 9). The IEF gel contained 2% (wt/vol) ampholytes composed of 40% pH 3.5–10 and 60% pH 6–8 Ampholines. MW, molecular weight.

IEF of the heavy and light chains of DR and Ia molecules has revealed that both subunits of a given specificity display microheterogeneity (15, 42-44). In this study and those cited above, the heterogeneity does not appear to be a result of artifactual modifications such as deamidation, carbamylation, or oxidation. The urea solutions used in this study were freshly prepared and deionized to remove cyanate ions which can cause carbamylation (45). The presence of the ampholytes also generally protects proteins from this type of modification (46). Carbamylation does not appear to occur when the gel pieces are equilibrated in urea and NP-40 because the DR heavy chain focuses as three to four bands even when this process is omitted. The proteins could be oxidized by the ammonium persulfate in the first dimension. However, actin focuses as two major bands as has been observed in O'Farrell 2-D gels (46) in which IEF is the first dimension and SDS-PAGE the second (28). The DR and Ia heavy and light chains also appear as multiple spots in O'Farrell 2-D gels (15, 42, 43). Oxidation in the IEF gel does not seem to occur because the protein standards focus as reported elsewhere (47) and prerunning the gel does not reduce the number of bands.

The basis of the microheterogeneity within a DRw specificity remains to be fully explained. Sialic acid heterogeneity accounts for some of the bands, but even after removal of most of the carbohydrate, the heavy and light chains each focus as two major bands. Accumulating evidence suggests that there may be a second DR-like antigen in man (48, 49). Several laboratories have mapped a second B-cell specific antigen to the right of the HLA-B locus, perhaps between the HLA-A and -B loci (49, 50). It is now being investigated whether the multiple bands observed for the DR subunits represent the products of multiple loci or post-translational or artifactual modifications of one product. One possibility is phosphorylation heterogeneity. It has been demonstrated that the HLA-A and -B heavy chains are phosphorylated *in vivo* (51) and it has also been shown that at least the DR heavy chain can be phosphorylated (40). As noted earlier, it is also possible that the large DR subunit may contain two types of oligosaccharide (41).

In addition to the heavy and light chains, DR immunoprecipitates contain two other polypeptides designated M1 and M2. It has not been determined whether these proteins are MHC-encoded or whether they are part of the native DR complex. Both proteins were invariant by IEF in all cell lines analyzed. One of the polypeptides may be identical to the one described by Giphart et al. (52). The M1 and M2 proteins do not appear to be directly recognized by the anti-29,34 serum because they can be largely dissociated from the DR complex by incubation at 37°C or by washing the SaCI-bound immunoprecipitate with 0.1% SDS which does not dissociate the rest of the immune complex. Studies using the cross-linking reagent dimethyl 3,3'-dithiobispropionimidate (19, 53) or tartryl bis(glycylhydrazine) (54) have shown that the DR and Ia antigens exist as a dimer of heavy and light chain in solution. Dancey et al. (53) have further shown that the murine I-E/C antigen exists as a dimer on the cell surface and is not cross-linked to other cellular components. These findings suggest that other proteins, such as M1 and M2, are not part of the DR complex. However, not all of the I-E/C antigens were cross-linked at the concentrations of DTBP used and extensively cross-linked proteins may not have been detergent solubilized (53). In addition, the gel systems used may not have resolved M1 from the heavy chain. The M1 protein may be an intracellular protein which associates with the DR complex after detergent solubilization because it is not precipitated with anti-p29,34 serum

when the antibody is incubated with intact cells (Fig. 2). An analogous protein has been described in the murine system (55). An invariant basic protein of 31,000 mol wt appeared in all immunoprecipitates of murine I-A and I-E/C subregion products. This protein, I_i , was not labeled with I^{125} using lactoperoxidase, also suggesting that it may be intracellular (55).

The human DR antigens have been demonstrated to be homologous in structure and function to the Ia antigens of the mouse and guinea pig. The strongest homology has been observed between the human DR and the murine I-E/C antigens in terms of serological cross-reactivity and (56, 57) and N-terminal amino acid sequence homology (1). Analyses of the tryptic peptides of I-E/C heavy (α) and light (β) chains demonstrated that most of the structural variability between different allelic products is in the β subunit (58-60). The β -chain from different genotypes shared on the average 50% of their peptides, whereas the α -chains shared 90% (58). The Ia antigens of mouse and guinea pig have also been analyzed by IEF (15, 16, 43, 44). As with the human DR antigens, the heavy chain was acidic whereas the light chain was neutral to basic. Both subunits of a given genotype and subregion appeared as multiple spots, but it was predominantly the light chain that showed structural polymorphism between genotypes (15). 2-D gel and peptide map analyses indicated that both I-E/C subunits are MHC-controlled, but although the α -chain maps to the I-E/C subregion, the β -chain maps to I-A (15, 61, 62). The I-A α - and β -chains both appear to map to the I-A subregion and exhibit considerable structural polymorphism by peptide map analysis (17, 63).

The data presented in this paper demonstrate that, analogous to the murine I-E/C antigens, the light chains of the human DR antigens are structurally polymorphic and appear to be controlled by the HLA-D/DR region. No polymorphism was detected among the DR heavy chains and it can not be concluded if they are MHC-encoded. The I-E/C heavy chains only showed limited differences as measured by peptide map analysis which tends to overestimate differences.

An antigen analogous to the murine I-A (or I-J) subregion antigens has not been identified in man. In this study, the nonglycosylated forms of the DR heavy and light chains each focused as two major bands which could indicate that there are two nonallelic DR antigens. However, other interpretations are possible. Further studies of the genetics, serology, and biochemistry of the HLA-D/DR region and its products should disclose if multiple loci do, in fact, exist.

Summary

Human HLA-DR antigens were immunoprecipitated from Nonidet P-40 extracts of [35 S]methionine-labeled B lymphoblastoid cell lines and compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). Two-dimensional (2-D) gel analyses, combining SDS-PAGE in the first dimension and IEF in the second dimension, revealed that the heavy (α) and light (β) chains of each DRw specificity displays microheterogeneity of charge. However, the pattern of the heavy chain did not vary among different DRw specificities. In contrast, the light chains of different DRw types varied both in apparent size and charge distribution. Removal of sialic acids with neuraminidase or inhibition of glycosylation with tunicamycin reduced the microheterogeneity of both DR subunits. However, the heavy and light chains each still focused as two major bands, suggesting

that other post-translational modifications contribute to the microheterogeneity or that there are two nonallelic DR-like molecules. After treatment with either neuraminidase or tunicamycin, the DR light chains, but not the heavy chains, were still structurally polymorphic. The DR light chains of serologically cross-reactive specificities displayed similar 2-D gel patterns suggesting that the structural polymorphism of the DR light chains is the basis for the serologically detected polymorphism of the HLA-DR antigens.

Two additional polypeptides were observed in immunoprecipitates of DR antigens. These proteins, designated M1 and M2, both had a basic isoelectric point and were invariant among different cell lines. The protein M1 may be intracellular because it can not be immunoprecipitated from the cell surface.

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